Circumventing antimicrobial-resistance and preventing its development in novel, bacterial infection-control strategies

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Autoantibody Detection for Diagnosis in Direct Immunofluorescence-Negative Mucous Membrane Pemphigoid

Ocular and Other Sites Compared

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Purpose: To assess whether a panel of serum pemphigoid autoantibody tests could be used to confirm an immunopathologic diagnosis of mucous membrane pemphigoid (MMP) in direct immunofluorescent negative (DIF−) MMP patients.

Design: Prospective cross-sectional study.

Participants: Seventy-six patients with multisite MMP with 45 matched control participants.

Methods: Enzyme-linked immunosorbent assays (ELISAs) for BP180 and BP230 (MBL International, Woburn, MA), immunoglobulin A (IgA) A and immunoglobulin G indirect immunofluorescence (IIF) on human salt-split skin and the keratinocyte footprint assay for anti−laminin 332 antibodies.

Main Outcome Measures: Sensitivity and specificity of autoantibody detection and significant differences for individual tests and test combinations for MMP involving different sites.

Results: All DIF− patients (24/73 [31.8%]) had either ocular-only disease or ocular involvement in multisite disease. Serum pemphigoid autoantibodies were detected in 29 of 76 MMP patients (38.2%) compared with 3 of 45 control participants (6.7%). Autoantibody reactivity detected by any 1 or more of the tests was present in 6 of 24 DIF− patients (25%) compared with 22 of 49 DIF positive (DIF+) patients (44.9%). Ocular-only MMP serum reactivity was not significantly different for any test or test combination compared with control participants, whereas DIF− multisite ocular MMP differed for 1 ELISA and 3 of 7 test combinations. By contrast, for DIF+ nonocular MMP patients, all the individual tests, apart from IgA IIF, and all test combinations were significantly different compared with those for control participants. For the entire MMP cohort, the sensitivity of all individual tests was low, having a maximum of 21.05% for BP180 reactivity but increasing to 38.16% for an optimal test combination. Disease activity was associated strongly with positive serologic findings.

Conclusions: Pemphigoid serum autoantibody tests did not provide immunopathologic evidence of MMP in ocular-only MMP patients but showed limited value in DIF− multisite ocular MMP patients. The requirement for immunopathologic confirmation of MMP by autoantibody detection is inappropriate for DIF− ocular-only MMP patients, resulting in missed diagnoses, delayed therapy, and poor outcomes. Alternative diagnostic criteria for ocular-only MMP are required to exclude the other causes of scarring conjunctivitis until more sensitive and specific immunopathologic tests become available. *Ophthalmology 2020;■:1–11 © 2020 by the American Academy of Ophthalmology*

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Mucous membrane pemphigoid (MMP) is an autoimmune subepidermal blistering disease. Autoantibodies are usually present and directed against different components of the epithelial basement membrane (BM) of the mucosal orifices, with or without skin involvement. All pemphigoid disorders with predominant involvement of mucous membranes are termed *MMP*.[1] Mucous membrane pemphigoid patients with lesions limited to ocular and oral sites have been termed *ocular-only MMP* patients, synonymous with pure ocular MMP[2] or oral-only MMP.[3] The conjunctiva is involved in two thirds of MMP patients.[1]

Mucous membrane pemphigoid diagnosis currently requires both clinical criteria and biopsy results showing immunoglobulin G (IgG), immunoglobulin A (IgA), or complement at the epithelial BM zone, indicating the presence of autoantibodies, using either direct immunofluorescence (DIF) or immunohistochemistry and immunoelectron microscopy. Direct immunofluorescence positive (DIF+) findings from

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any 1 site are accepted as diagnostic immunopathologic evidence for disease at any other site that meets the clinical criteria for MMP.[1] Biopsy samples for DIF are obtained from perilesional tissue of affected sites,[1] including, where possible, uninflamed conjunctiva, but biopsy samples from clinically unaffected sites may also show positive results.[4] However, biopsy samples cannot always be obtained for DIF (consent may be declined or conjunctiva may be inaccessible in advanced ocular disease) and, furthermore, are less sensitive in ocular-only MMP than for MMP at other sites,[5–7] with positive results found in only approximately 50% of patients, despite the use of multiple biopsy samples.[3,8] When negative DIF (DIF−) results are found or results are unavailable, the detection of circulating epithelial basement membrane autoantibodies in serum can be used to confirm the diagnosis.[1] In MMP, 6 target antigens have been recognized as pemphigoid autoantibodies, including BP180 (also termed *collagen type XVII*), BP230, and laminin 332, for which tests are widely available.[9–14] Pemphigoid autoantibodies have been detectable in variable proportions of MMP patients, from as low as 10% for IIF salt-split skin (SSS), to 0% for immunoblotting or immunoprecipitation for BP180 and BP230 in a subset of 10 ocular-only MMP patients,[15] to as high as 84%[16] for MMP patients having mixed site involvement.

Our primary hypothesis was that a panel of serum pemphigoid autoantibody tests and their combinations might be used to confirm an immunopathologic diagnosis of MMP in DIF− patients with ocular involvement. The hypothesis was tested by evaluating the sensitivity and specificity of tests and their combinations for patients with that of age-, gender-, and race-matched control participants.

Methods

The study was approved by the UK Research Ethics Service (reference no., 09/H0721/54) and adhered to the tenets of the Declaration of Helsinki. This was a prospective cross-sectional study of patients diagnosed with MMP and an age-, gender-, and race-matched control population, all of whom donated blood for these serologic studies. Patients and control participants provided informed consent and were recruited between December 21, 2009, and August 5, 2011.

Patients

Mucous membrane pemphigoid patients were recruited from among both existing patients and new referrals at 2 London clinics, the Corneal and External Disease Clinic of Moorfields Eye Hospital NHS Foundation Trust and the Oral Medicine and Dermatology Clinics of Guys and St. Thomas's NHS Foundation Trust. The results of previous DIF tests were recorded, and if these had not been carried out, a biopsy sample was obtained and processed for DIF using standard techniques.[17] The diagnosis of MMP for patients with ocular involvement, without positive DIF results, was based on the clinical and pathologic criteria that we proposed previously for this subset of patients.[3,8,18] Data were collected using a case report form designed for this study.[3] A history was obtained from all MMP patients, focusing on previous involvement of sites of MMP and general health, and all MMP patients underwent an examination for signs of MMP at all potential anatomic sites, apart from the esophagus, by ophthalmologists, a dermatologist, an oral medicine specialist, and otolaryngologists. Some patients declined the additional examinations for screening of extraocular sites (13 oral, 14 skin, 37 nasopharyngeal, 15 genital, and 16 perianal). The history of disease at all sites was used to classify patients by site of involvement, both those whose disease was in remission with no residual clinical signs (common in oral MMP) and when the additional examinations had been declined. The sites assessed for involvement by MMP and screening criteria for involvement at these sites have been described and tabulated.[3]

Control Participants

The number of control participants in this study (n = 45) was chosen a priori to give an 80% power to detect a difference in the proportions of BP180-NC16a autoantibodies. This was calculated using Wieland's data on age, 14 of 337 (4.15%) gender-stratified control participants having detectable levels,[19] and our pilot data from our MMP patients showing 8 of 32 patients (25%) had detectable levels. Age-, gender-, and race-matched control participants were recruited from among healthy staff and patients who were undergoing surgery for ocular conditions, without associated systemic disease.

Serologic Tests

The serology test results analyzed in this study were duplicated in a service laboratory in 2014 and 2015 and in the laboratory of the Centre for Blistering Diseases, The University of Groningen, in 2018 and 2019; discrepancies between the 2 sites were retested at the St. John's Institute of Dermatology Laboratory in 2019. This was carried out to resolve the issue of unreliable data provided by the service laboratory that became apparent in 2018. The sera were stored at the UCL Institute of Ophthalmology, London, at −80° C until March 2018 and at −20° C thereafter. Laboratory staff were masked to the clinical findings.

Table 1 describes the 5 tests carried out on the sera for all 76 patients and 45 control participants. The 51 discrepancies between the Groningen and service laboratory results were retested by St. John's. For the 45 patients for which the Groningen results were confirmed by St. John's, the Groningen results were used for the analysis. The service laboratory findings were used for the remaining 6 tests after the discrepancies with Groningen were confirmed by 2 repeat tests at St. John's. Laminin 332 reactivity was reported only for the Groningen keratinocyte footprint assay[20] results. Indirect immunofluorescence was carried out using human SSS, although protocols varied because no standards exists for this test.[21] The MBL International (Woburn, MA) enzyme linked immubosorbent assays (ELISAs) were carried out according to the manufacturer's protocol, but procedures differed between these laboratories with regard to the reporting of the results; at Groningen, sera with ELISA results of 6 U/ml or more were retested up to twice more, and the results scored as positive if at least 2 tests met the manufacturer's recommended cutoff of 9 U/ml or more and were scored as negative if any 2 tests showed lower concentrations than this. At the service laboratory, participants with results of 9 U/ml or more were recorded as positive unless a test showed only weakly positive results when it was repeated and were reported as positive when the repetition was positive, or the results were recorded as negative if the repeat test showed negative results. At St. John's, results were reported as positive when the results met the manufacturer's recommended cutoff of 9 U/ml or more.

Statistical Analysis

The sensitivity and specificity were computed for those autoantibody tests showing a significantly higher frequency of positive

Table 1. Descriptions of the 5 Tests Used to Detect Serum Pemphigoid Autoantibodies in Mucous Membrane Pemphigoid Patients, the Proportions of Positive Results Compared with Those of Control Participants, Sensitivity, Specificity, and Youden's Index

Test No.*	Antigens and Substrates	Test Methodology[†] [‡]	Positive Reactions, No. (%)			Sensitivity and Specificity		
			Mucous Membrane Pemphigoid Patients (n = 76)	*Control Participants (n = 45)*	**Exact *P* Value**	*Sensitivity (%)*	*Specificity (%)*	*Youden's Index*
1	ELISA BP180-NC16a MBL	Enzyme-linked immunosorbent assays (MBL International), cutoff < 9 U/ml	16 (21.05)	2 (4.44)	**0.016**	21.05	95.56	16.61
2	ELISA BP230 MBL		10 (13.16)	1 (2.22)	0.052	13.16	97.78	10.94
3	IgA IIF SSS	Indirect immunofluorescence on human 1-molar SSS	5 (6.58)	0 (0.00)	0.156	6.58	100	6.58
4	IgG IIF SSS		9 (11.84)	0 (0.00)	**0.026**	11.84	100	11.84
5	KFA	Indirect immunofluorescence laminin 332 assays: KFA (Groningen) for laminin 332 antibody detection	3 (3.95)	0 (0.00)	0.233	3.95	100	3.95
Combined reactions								
1 + 2			19 (25.00)	3 (6.67)	**0.014**	25.00	93.33	18.33
3 + 4			13 (17.11)	0 (0.00)	**0.002**	17.11	100	17.11
1 + 3 + 4			25 (32.89)	2 (4.44)	**< 0.001**	32.89	95.56	28.45
1 + 2 + 3 + 4			26 (34.21)	3 (6.67)	**< 0.001**	34.21	93.33	27.54
1 + 3 + 4 + 5			28 (36.84)	2 (4.44)	**< 0.001**	36.84	95.56	32.40
2 + 3 + 4 + 5			23 (30.26)	1 (2.22)	**< 0.001**	30.26	97.78	28.04
1 + 2 + 3 + 4 + 5			29 (38.16)	3 (6.67)	**< 0.001**	38.16	93.33	31.49

ELISA = enzyme-linked immunosorbent assay; IgA = immunoglobulin A; IgG = immunoglobulin G; IIF = indirect immunofluorescence; KFA = Keratinocyte footprint assay; SSS = salt-split skin.
Statistically significant *P* values were defined as <0.05 and have been identified in boldface.
*Test numbering is used in Figures 1–3.
[†]All tests were from 76 patients and 45 control participants, apart from the BP230 ELISA, which were performed on only 60 of 76 MMP patients at the service laboratory.
[‡]Commercially available tests were carried out according to the manufacturer's instructions. Indirect immunofluorescence was carried out as previously described.[31]

Table 2. Clinical Characteristics of Mucous Membrane Pemphigoid Patients and Control Participants, Direct Immunofluorescence Results, and Serum Pemphigoid Autoantibody Test Results for All Participants and Participants with Both Limited-Site and Multiple-Site Involvement

Demographics	All Mucous Membrane Pemphigoid Patients	Mucous Membrane Pemphigoid Patients Categorized by Different Sites of Involvement*							Control Participants
		Ocular Only	*Ocular and Oral Only*	*Oral Only*	*All Nonocular*	*Nasopharyngeal and Any Other*	*Genital and Any Other*	*Skin and Any Other*	
No. (%)	76	18	15	14	20	16	8	14	45
Male gender, no. (%)	38 (50)	9 (50)	11 (73.3)	5 (35.7)	5 (25.0)	6 (37.5)	2 (25.0)	8 (57.1)	22 (49)
Age (yrs)									
Mean (standard deviation)	59.9 (14.6)	63.2 (18.0)	55.4 (10.5)	61.1 (10.0)	63.7 (9.5)	57.4 (17.7)	66.25 (7.8)	57.4 (15.2)	61.4 (13.1)
Range	18–83	24–83	38–75	47–81	47–81	18–78	56–76	23–74	18–86
White race, no. (%)†	64 (91.4)	16 (88.9)	13 (100)	11 (91.7)	15 (83.3)	14 (93.3)	5 (71.4)	11 (84.6)	42 (93)
Race not declared, no.‡	6	0	2	2	3	1	1	1	0
Systemic immunotherapy, no. (%)	43 (56.6)	13 (72.2)	9 (60.0)	3 (21.4)	5 (25.0)	12 (75.0)	3 (37.5)	8 (57.1)	None
Direct immunofluorescence results, no. (%)									Not performed
Positive	49 (67.1)	6 (35.3)	12 (80)	13 (100)	19 (100)	11 (68.7)	4 (57.1)	9 (64.3)	
Negative (all ocular)§	24 (32.9)	11 (64.7)	3 (20)	0 (0.0)	0 (0.0)	5 (31.3)	3 (42.9)	5 (35.7)	
Unknown‡	3	1	0	1	1	0	1	0	
Serum autoantibody results, no. (%)									
Any positive	29 (38.2)	3 (16.7)	5 (33.3)	9 (64.3)	13 (65.0)	7 (43.8)	6 (75.0)	5 (35.7)	3 (6.7)
Positive in DIF+	22/49 (44.9)	1/6 (16.7)	5/12 (41.7)	8/13 (61.5)	12/19 (63.2)	5/11 (45.5)	3/4 (75.0)	3/9 (33.3)	Not applicable
Positive in DIF−	6/24 (25.0)	2/11 (18.2)	0/3 (00.0)	0/0	0/0	2/5 (40.0)	3/3 (100.0)	2/5 (40.0)	

DIF+ = direct immunofluorescent positive; DIF− = direct immunofluorescent negative.
*Nonocular, nasopharyngeal, genital, and skin categories are not mutually exclusive.
†Numbers and percentages are for white races: mucous membrane pemphigoid patients additionally included 2 Asian persons, 1 Black person, and 3 persons of other races; control participants additionally included 2 Asian persons and 1 Black person.
‡Missing values for race and direct immunofluorescent results are shown. These were excluded from the denominators for calculation of percentages.
§Direct immunofluorescent-negative patients all showed ocular involvement: 11 of 24 (45.8%) showed ocular-only involvement, 3 of 24 (12.5%) showed ocular and oral involvement only, and the remaining 10 of 24 (41.6%) showed ocular involvement with the other nonocular sites (nasopharyngeal, genital, and skin).

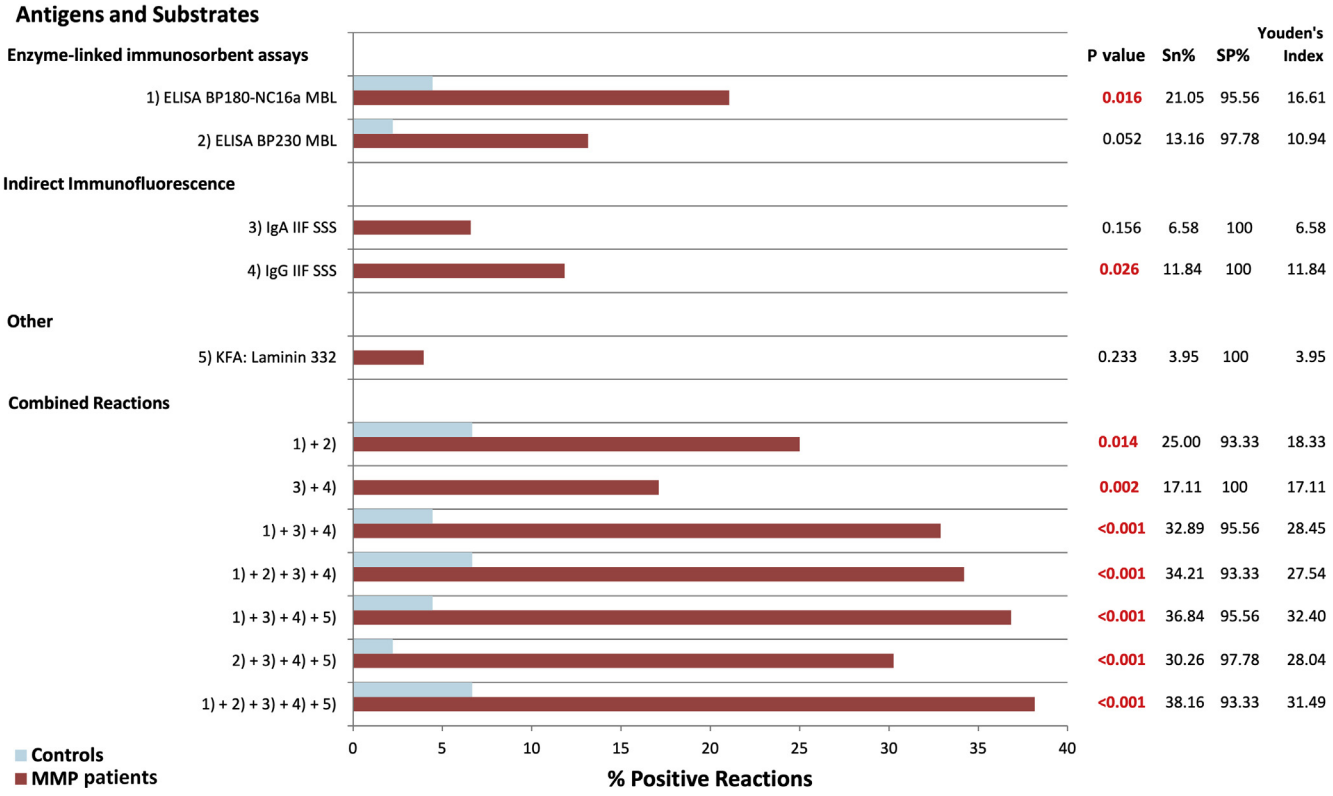

Figure 1. Bar graph comparing serum pemphigoid autoantibody detection test results for all mucous membrane pemphigoid (MMP) patients (n = 76) with those of all control participants (n = 45). Individual tests are numbered 1 through 5, and test combinations are referred to by these numbers. The numbers from which the percentages are derived are given in Table 1. Youden's index is shown only when a significant (or borderline) difference exists in the percentage of positive reactions. *P* values of 0.05 or less are highlighted in red. ELISA = enzyme-linked immunosorbent assay; IgA = immunoglobulin A; IgG = immunoglobulin G; IIF = indirect immunofluorescence; Sn = sensitivity; Sp = specificity; SSS = salt-split skin.

reactions in MMP patients compared with control participants. Youden's index (sensitivity% + specificity% − 100) was used to identify the best diagnostic test, giving equal weight to specificity and sensitivity and taking the clinical diagnosis of MMP as the reference standard. Youden's index of 100% indicates a perfect diagnostic test and more than 80% is an acceptable value for a good test. The above procedures were repeated for some combinations of different tests, whereby the serologic results were regarded as positive when 1 or more tests of the combination showed a positive reaction. The aim was to explore combinations that improved sensitivity or specificity or gave a higher Youden's index. The frequency of positive reactions in control participants and in both DIF+ and DIF− MMP patients were also compared using the Fisher exact test. The frequency of positive reactions in control participants and MMP clinical phenotypes (MMP involving different combinations of sites) were compared using the Fisher exact test, as appropriate.

Results

Characteristics of Mucous Membrane Pemphigoid Patients and Control Participants

Table 1 describes the serologic tests and the results of both individual tests and test combinations for all patients combined compared with control participants. Table S1 (available at www.aaojournal.org) provides full clinical and serologic data for the individual patients and control participants. This dataset is also available as an Excel Workbook (Microsoft, Redmond, WA) at Mendeley Data (https://data.mendeley.com/datasets/7pxbkx84r3/1) including the patient and control dataset in sheet 1 and serologic results from all 3 laboratories in sheet 2. Table 2 summarizes the demographic data and overall positive serologic test results for patients with different sites of MMP involvement and by DIF status. Mucous membrane pemphigoid patients and control participants were similar in terms of age, gender, and race distribution.

Direct Immunofluorescence

A DIF result was available for 73 of 76 MMP patients. Direct immunofluorescence showed positive results for at least 1 site in 49 of 73 patients (67.1%). We included the 24 patients with negative DIF results and the 3 for whom these results were not available but who met our clinical criteria for a diagnosis of MMP.[3] All 24 DIF− patients showed ocular involvement (Table 2).

Serum Pemphigoid Autoantibody Tests

When all tests were evaluated for patients and control participants, at least 1 positive test result was reported for 29 of 76 MMP patients (38.2%); 22 of 49 (44.9%) direct IIF-positive patients showed positive results versus 6 of 24 (25%) DIF− patients (Table 2).

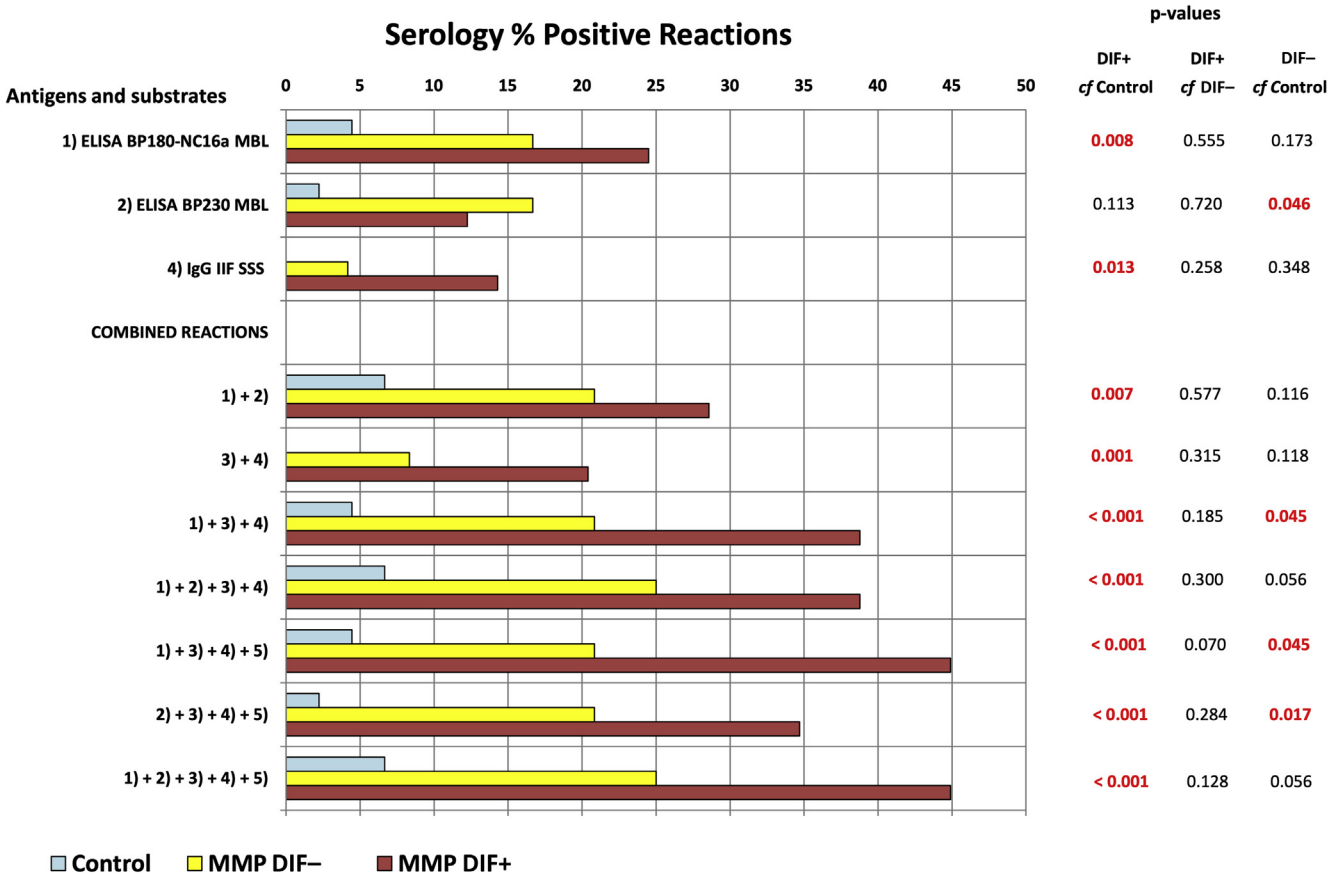

Figure 2. Bar graph comparing serum pemphigoid autoantibody detection test results from direct immunofluorescence-positive (DIF+) patients (n = 49) and direct immunofluorescence-negative (DIF−) patients (n = 24). Individual tests are numbered 1 through 5: (1) enzyme-linked immunosorbent assay (ELISA) BP180-NC16a MBL, (2) ELISA BP230 MBL, (3) immunoglobulin A (IgA) indirect immunofluorescence (IIF) salt-split skin (SSS), (4) immunoglobulin G (IgG) IIF SSS, and (5) keratinocyte footprint assay (KFA). Tests 3 and 5 were not analyzed for differences between DIF+ and DIF− patients because of small numbers. Percentages are given here for which the numbers are provided in Table S2. *P* values of 0.05 or less are highlighted in red. *cf* = compared with.

Proportions of Mucous Membrane Pemphigoid Patients and Control Participants with Positive Serum Pemphigoid Autoantibody Results for Individual Tests and Test Combinations

For individual tests results for the entire patient group (Table 1), only ELISA BP180-NC16a MBL and IgG IIF SSS results were significantly different from those of control participants. Control sera showed positive results in 2 tests: 2 of 45 samples (4.44%) for the ELISA BP180-NC16a MBL and 1 of 45 samples for the ELISA BP230 MBL. These findings are shown graphically in Figure 1. Test combinations (any 1 or more positive test results) showed substantially higher sensitivities than any individual test, with similar specificities, although sensitivities were still low (17.1%–38.2%), contributing to a low Youden's index. The ELISA BP180-NC16a MBL, IIF on SSS for IgG and IgA, and laminin 332 assay were an optimal combination, with a sensitivity of 36.8 and specificity of 95.56. When all 5 tests were combined, the sensitivity rose slightly to 38.16 but with a slightly reduced specificity of 93.33 because 1 control sample showed positive results for BP230.

Table S2 (available at www.aaojournal.org) is expanded from Table 1 to include the serologic test results for the following additional patient subsets compared with control participants: DIF+ and DIF− patients, the sites most frequently involved by MMP (ocular only, oral only, ocular and oral only, and all nonocular sites), and results for DIF+ nonocular patients. The latter group was chosen because of our unanticipated finding showing that ocular-only patients and DIF− ocular patients with multisite involvement included a lower proportion of patients with detectable pemphigoid autoantibodies. These results are illustrated in Figure 2, showing the test reactivity for the comparison of DIF− and DIF+ patients compared with control participants, and in Figure 3, showing the test reactivity for the following different MMP phenotypes: ocular only, oral only, ocular and oral only, and all nonocular sites of involvement.

Proportions of Patients with Positive Serologic Results with and without Active Inflammation, Systemic Immunosuppression, or Both

Table S3 (available at www.aaojournal.org), for patients with oral or ocular MMP or both (n = 74), shows a strong association with disease activity but not with immunosuppression, probably because 32 of 43 immunosuppressed patients (74.4%) still had active inflammation.

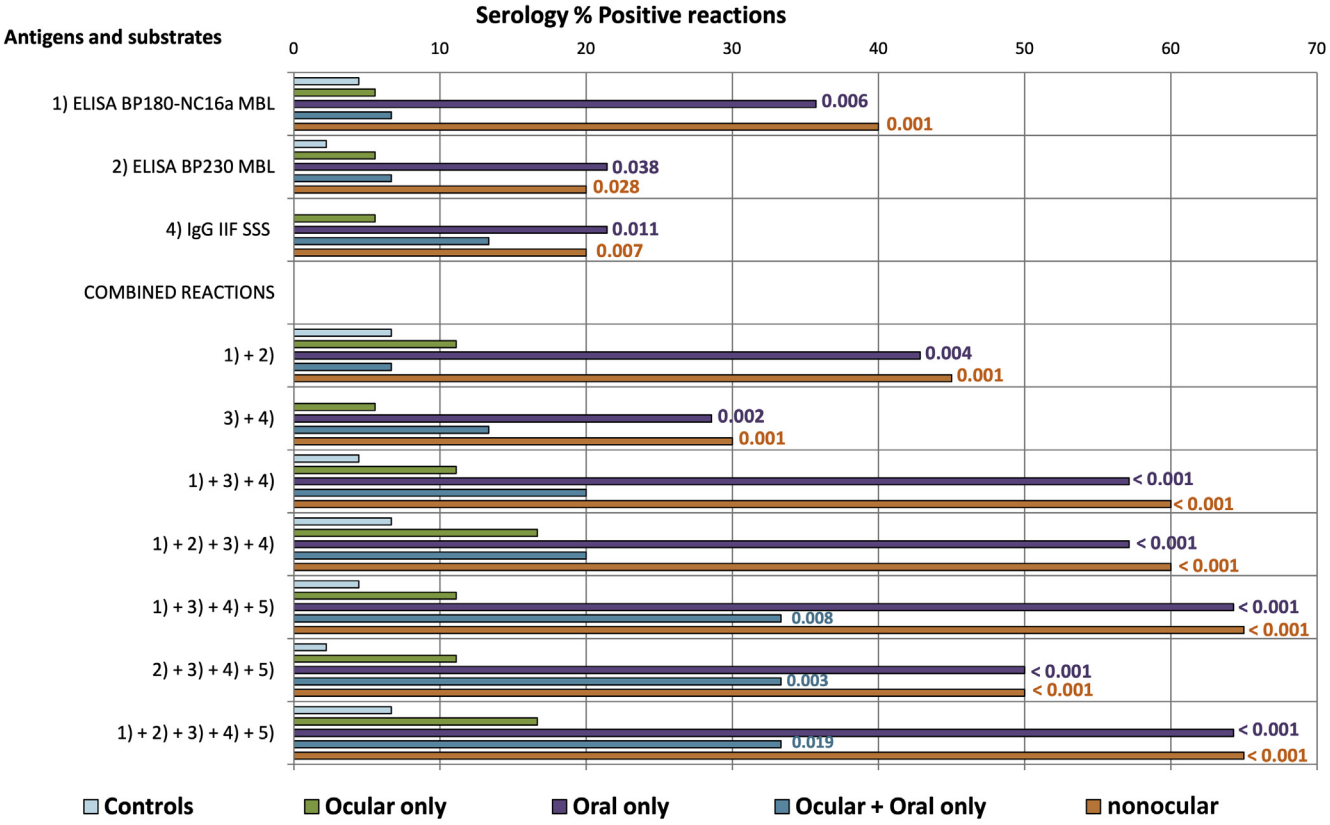

Figure 3. Bar graph comparing serum pemphigoid autoantibody detection test results from mucous membrane pemphigoid (MMP) phenotypes defined by MMP site involvement with those from control participants. Individual tests are numbered 1 through 5: (1) enzyme-linked immunosorbent assay (ELISA) BP180-NC16a MBL, (2) ELISA BP230 MBL, (3) immunoglobulin A (IgA) indirect immunofluorescence (IIF) salt-split skin (SSS), (4) immunoglobulin G (IgG) IIF SSS, and (5) keratinocyte footprint assay (KFA). *P* values of 0.05 or less (exact 2-sided) are shown at end of bars, each compared with controls. Percentages are used here for which the numbers are provided in Table S2.

Proportions of Direct Immunofluorescence-Positive and -Negative Patients with Positive Serum Basement Membrane Autoantibody Reactivity for Individual and Test Combinations

Figure 2 and Table S2 show that, with 3 exceptions, DIF+ patients showed significantly different (more often positive) serologic findings both for single tests and all test combinations compared with control participants. Patients with negative DIF results with positive BP230 ELISA results, BP180-NC16a/IIF SSS combination results, or combinations of ELISAs, IIF SSS, and laminin 332 assay results were significantly different from control participants (Fig 2), although the sensitivity is low for these tests (Fig 1).

Proportions of Patients with Ocular-Only, Oral-Only, Ocular- and Oral-Only, and All Nonocular Sites Involved by Mucous Membrane Pemphigoid with Positive Serum Basement Membrane Autoantibody Reactivity for Individual Tests and Test Combinations

In ocular-only patients, only 1 of 6 DIF+ patients showed positive serum test results, as opposed to 12 of 19 DIF+ nonocular patients (Table 2), suggesting that there may be lower levels of detectable autoantibodies in ocular disease patients independent of DIF status. Figure 3 and Table S2 show that for ocular-only MMP sites of involvement, no significant difference was found in test reactivity compared with control participants for both individual tests and any test combinations. This finding was similar but less extreme for patients with both ocular- and oral-only involvement (n = 15) for whom no individual test results were significant. For all DIF− patients (n = 24), positive BP230 ELISA (4/24) results were significantly different, as were test combinations including at least 1 positive ELISA result, 1 positive IIF SSS (6/24) result, or both in patients compared with control participants. Conversely for pure oral involvement and any patients with nonocular site involvement (all but one of whom was DIF+), test reactivity was significantly different from control participants for both ELISAs and IgG SSS, as well as all test combinations.

Discussion

This cross-sectional study of 76 patients with a clinical diagnosis of MMP included 24 (32.9%) who were DIF− but who met clinical and pathologic criteria for DIF− MMP with ocular involvement[3,8,18,22,23] and included 18 patients with ocular-only MMP (6/18 DIF+). To our knowledge, this is the largest study of ocular-only MMP studied to date.[2,15] Serum pemphigoid autoantibodies were detected in

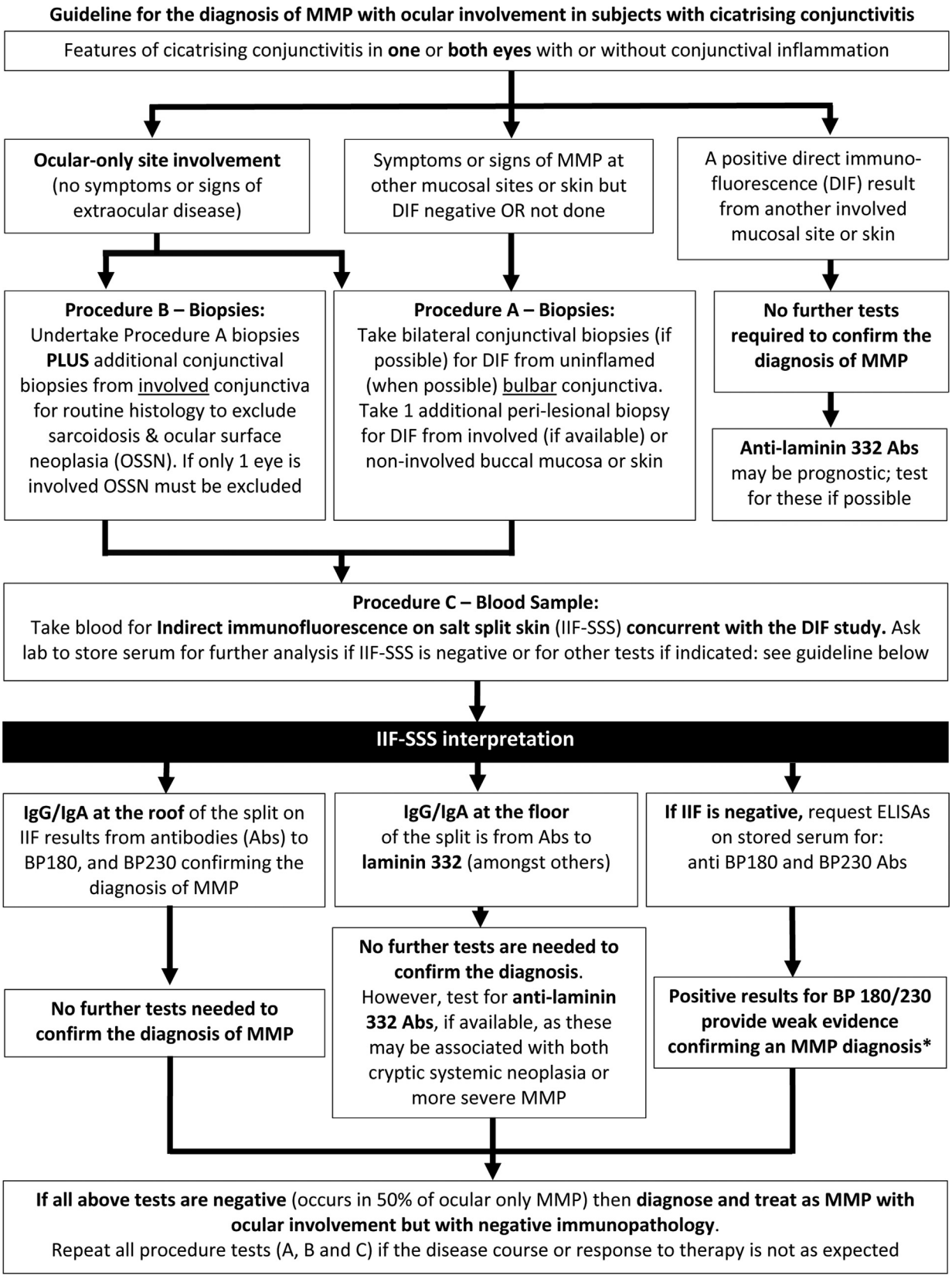

Figure 4. Guideline showing a test strategy for the diagnosis of MMP with ocular involvement in subjects with cicatrising conjunctivitis. ELISA = enzyme linked immunosorbent assay; IgA = immunoglobulin A; IgG = immunoglobulin G; MMP = mucous membrane pemphigoid.

29 of 76 MMP patients (38.2%) compared with 3 of 45 control participants (6.7%) in whom positive results were found only for ELISAs. The proportions of autoantibodies detected in DIF+ MMP patients was higher at 22 of 49 patients (44.9%) compared with DIF− MMP at 6 of 24 patients (25%). Laminin 332 assay results were positive in 3 DIF+ MMP patients. Serologic results were more often positive in patients with active inflammation.

Our primary hypothesis was that a panel of serum pemphigoid autoantibody tests might be used to confirm an immunopathologic diagnosis of MMP in DIF− patients with ocular MMP involvement. All DIF− patients showed ocular involvement. For DIF− patients, the only serologic test that was significantly different in patients compared with control participants was that for BP230 reactivity (positive in 4 of 24 patients). However, a test combination including at least 1 positive ELISA result and 1 positive IIF SSS result increased the proportion of positive test results (6/24 tests) and was significantly different from control participants (Table S2; Fig 2) but with low sensitivity (approximately 30%). For ocular-only MMP (n = 18), only 3 of 90 tests showed positive results, not significantly different from that for control participants. In summary, we found only limited support for our primary hypothesis by finding that this panel of widely available serologic tests do not contribute to the immunopathologic diagnosis of ocular-only MMP, although they are of limited value in DIF− MMP multisite ocular disease. It is unsurprising that patients who do not have antibodies at the epithelial BM (DIF−) that are probably deposited from the circulation are also less likely to have detectable circulating antibodies. Our findings for ocular-only MMP confirm those of 2 other studies of a total of 16 patients.[2,15]

One potential shortcoming of this study may result from antibody degradation resulting from the storage methodology and the time between sample collection and analysis. We think this unlikely because antibody function in serum stored at −20° C to −80° C is recommended for up to 10 years[24] and has been shown to be stable for this period[25] and because our ELISAs more often showed positive results when duplicate sera were retested in Groningen and St. John's 4 to 5 years after initial testing at the service laboratory. Another shortcoming may relate to misclassification of our ocular-only MMP patients. We believe this unlikely given that the strict criteria we have used recently became well established and coupled with the recognition that DIF and serologic findings may be negative in ocular MMP.[3,8,18,22,23] Our serologic results are compared with those of 13 similar MMP autoantibody studies in Table S4A (available at www.aaojournal.org)[2,13,16,26–35] and with 3 studies of control populations in Table S4B.[19,36,37] Our findings for BP180 and BP230 ELISAs, laminin 332, and IgA IIF SSS are comparable, whereas our proportions of participants showing positive IgG IIF SSS results are among the lowest reported. Differences in the proportions of routine tests that show positive results relate both to differences in disease activity and in serum reactivity for MMP involving different anatomic sites, as we have shown in this study, with both quiescent disease and ocular sites having lower reactivity.

Strengths of this study are that it is a prospective hypothesis-driven cross-sectional study for which participants were diagnosed and phenotyped using previously agreed-on criteria and that used serologic tests available in most dermatology immunopathology laboratories. Our results were duplicated in 2 independent laboratories, and discrepancies were verified in a third. Our finding of 51 of 468 discrepancies (10.9%) for duplicate testing, of which only 6 from 1 laboratory could be confirmed, shows that interpretation of results requires confidence in the quality standards of the laboratory being used. The study is also unique (Table S4, available at www.aaojournal.org) in including, at the time of blood sampling, disease activity scores, immunosuppression data, a control population, and serum storage data.

The findings from this study on the value of circulating autoantibody tests for the immunopathologic diagnosis of MMP concur with those of previous studies on the poor sensitivity of DIF in MMP with ocular involvement and the need for an alternative diagnostic strategy for ocular disease. Given the low sensitivity of serologic tests in MMP and the false-positive rate in control participants, the finding of positive results must be interpreted with caution before using these as confirmation of a diagnosis of MMP. Our recommendation for a diagnostic guideline for potential cases of MMP with ocular involvement arising from these studies is in Figure 4. Our studies also have implications for the development of diagnostic tests and the pathogenesis of MMP. Either current immunopathologic tests are too insensitive for the detection of low levels of tissue-fixed or circulating antibodies or a subset of MMP patients exists in whom an alternative, possibly cell-mediated, immunopathologic reaction directed at the epithelial BM epitopes is predominant.[8] Novel tests for MMP are required that may include cellular, cytokine, or gene expression biomarkers for MMP.

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References

1. Chan LS, Ahmed AR, Anhalt GJ, et al. The first international consensus on mucous membrane pemphigoid: definition, diagnostic criteria, pathogenic factors, medical treatment, and prognostic indicators. *Arch Dermatol*. 2002;138(3):370−379.
2. Jonkman MF, Groot AC, Slegers TP, et al. Immune diagnosis of pure ocular mucous membrane pemphigoid: indirect immunofluorescence versus immunoblot. *Eur J Dermatol*. 2009;19(5):456−460.
3. Ong HS, Setterfield JF, Minassian DC, et al. Mucous membrane pemphigoid with ocular involvement: the clinical phenotype and its relationship to direct immunofluorescence findings. *Ophthalmology*. 2018;125(4):496−504.
4. Grau AE, Setterfield J, Saw VP. How to do conjunctival and buccal biopsies to investigate cicatrising conjunctivitis: improving the diagnosis of ocular mucous membrane pemphigoid. *Br J Ophthalmol*. 2013;97(4):530−531.

5. Thorne JE, Anhalt GJ, Jabs DA. Mucous membrane pemphigoid and pseudopemphigoid. *Ophthalmology*. 2004;111(1):45–52.
6. Bernauer W, Elder MJ, Leonard JN, et al. The value of biopsies in the evaluation of chronic progressive conjunctival cicatrisation. *Graefes Arch Clin Exp Ophthalmol*. 1994;232(9):533–537.
7. Leonard JN, Hobday CM, Haffenden GP, et al. Immunofluorescent studies in ocular cicatricial pemphigoid. *Br J Dermatol*. 1988;118(2):209–217.
8. Dart JK. The 2016 Bowman Lecture. Conjunctival curses: scarring conjunctivitis 30 years on. *Eye (Lond)*. 2017;31(2):301–332.
9. Domloge-Hultsch N, Gammon WR, Briggaman RA, et al. Epiligrin, the major human keratinocyte integrin ligand, is a target in both an acquired autoimmune and an inherited subepidermal blistering skin disease. *J Clin Invest*. 1992;90(4):1628–1633.
10. Bernard P, Prost C, Durepaire N, et al. The major cicatricial pemphigoid antigen is a 180-kD protein that shows immunologic cross-reactivities with the bullous pemphigoid antigen. *J Invest Dermatol*. 1992;99(2):174–179.
11. Roh JY, Yee C, Lazarova Z, et al. The 120-kDa soluble ectodomain of type XVII collagen is recognized by autoantibodies in patients with pemphigoid and linear IgA dermatosis. *Br J Dermatol*. 2000;143(1):104–111.
12. Tyagi S, Bhol K, Natarajan K, et al. Ocular cicatricial pemphigoid antigen: partial sequence and biochemical characterization. *Proc Natl Acad Sci U S A*. 1996;93(25):14714–14719.
13. Balding SD, Prost C, Diaz LA, et al. Cicatricial pemphigoid autoantibodies react with multiple sites on the BP180 extracellular domain. *J Invest Dermatol*. 1996;106(1):141–146.
14. Bhol KC, Goss L, Kumari S, et al. Autoantibodies to human alpha6 integrin in patients with oral pemphigoid. *J Dental Res*. 2001;80(8):1711–1715.
15. Chan LS, Yancey KB, Hammerberg C, et al. Immune-mediated subepithelial blistering diseases of mucous membranes. Pure ocular cicatricial pemphigoid is a unique clinical and immunopathological entity distinct from bullous pemphigoid and other subsets identified by antigenic specificity of autoantibodies. *Arch Dermatol*. 1993;129(4):448–455.
16. Setterfield J, Shirlaw PJ, Kerr-Muir M, et al. Mucous membrane pemphigoid: a dual circulating antibody response with IgG and IgA signifies a more severe and persistent disease. *Br J Dermatol*. 1998;138(4):602–610.
17. Zillikens D. Diagnosis of autoimmune bullous skin diseases. *Clin Lab*. 2008;54(11–12):491–503.
18. Saw VP, Dart JK. Ocular mucous membrane pemphigoid: diagnosis and management strategies. *Ocul Surf*. 2008;6(3):128–142.
19. Wieland CN, Comfere NI, Gibson LE, et al. Anti-bullous pemphigoid 180 and 230 antibodies in a sample of unaffected subjects. *Arch Dermatol*. 2010;146(1):21–25.
20. Giurdanella F, Nijenhuis AM, Diercks GFH, et al. Keratinocyte footprint assay discriminates antilaminin-332 pemphigoid from all other forms of pemphigoid diseases. *Br J Dermatol*. 2020;182(2):373–381.
21. van Beek N, Rentzsch K, Probst C, et al. Serological diagnosis of autoimmune bullous skin diseases: prospective comparison of the BIOCHIP mosaic-based indirect immunofluorescence technique with the conventional multi-step single test strategy. *Orphanet J Rare Dis*. 2012;7:49.
22. Tauber J. Ocular cicatricial pemphigoid. *Ophthalmology*. 2008;115(9):1639–1640. author reply 1640–1631.
23. Labowsky MT, Stinnett SS, Liss J, et al. Clinical implications of direct immunofluorescence findings in patients with ocular mucous membrane pemphigoid. *Am J Ophthalmol*. 2017;183:48–55.
24. Hendriks J, Stals C, Versteilen A, et al. Stability studies of binding and functional anti-vaccine antibodies. *Bioanalysis*. 2014;6(10):1385–1393.
25. Dard C, Bailly S, Drouet T, et al. Long-term sera storage does not significantly modify the interpretation of toxoplasmosis serologies. *J Microbiol Methods*. 2017;134:38–45.
26. Murakami H, Nishioka S, Setterfield J, et al. Analysis of antigens targeted by circulating IgG and IgA autoantibodies in 50 patients with cicatricial pemphigoid. *J Dermatol Sci*. 1998;17(1):39–44.
27. Leverkus M, Schmidt E, Lazarova Z, et al. Antiepiligrin cicatricial pemphigoid: an underdiagnosed entity within the spectrum of scarring autoimmune subepidermal bullous diseases? *Arch Dermatol*. 1999;135(9):1091–1098.
28. Schmidt E, Skrobek C, Kromminga A, et al. Cicatricial pemphigoid: IgA and IgG autoantibodies target epitopes on both intra- and extracellular domains of bullous pemphigoid antigen 180. *Br J Dermatol*. 2001;145(5):778–783.
29. Setterfield J, Theron J, Vaughan RW, et al. Mucous membrane pemphigoid: HLA-DQB1*0301 is associated with all clinical sites of involvement and may be linked to antibasement membrane IgG production. *Br J Dermatol*. 2001;145(3):406–414.
30. Carrozzo M, Cozzani E, Broccoletti R, et al. Analysis of antigens targeted by circulating IgG and IgA antibodies in patients with mucous membrane pemphigoid predominantly affecting the oral cavity. *J Periodontol*. 2004;75(10):1302–1308.
31. Oyama N, Setterfield JF, Powell AM, et al. Bullous pemphigoid antigen II (BP180) and its soluble extracellular domains are major autoantigens in mucous membrane pemphigoid: the pathogenic relevance to HLA class II alleles and disease severity. *Br J Dermatol*. 2006;154(1):90–98.
32. Calabresi V, Carrozzo M, Cozzani E, et al. Oral pemphigoid autoantibodies preferentially target BP180 ectodomain. *Clin Immunol*. 2007;122(2):207–213.
33. Bernard P, Antonicelli F, Bedane C, et al. Prevalence and clinical significance of anti-laminin 332 autoantibodies detected by a novel enzyme-linked immunosorbent assay in mucous membrane pemphigoid. *JAMA Dermatol*. 2013;149(5):533–540.
34. Hayakawa T, Furumura M, Fukano H, et al. Diagnosis of oral mucous membrane pemphigoid by means of combined serologic testing. *Oral Surg Oral Med Oral Pathol Oral Radiol*. 2014;117(4):483–496.
35. Cozzani E, Di Zenzo G, Calabresi V, et al. Autoantibody profile of a cohort of 78 Italian patients with mucous membrane pemphigoid: correlation between reactivity profile and clinical involvement. *Acta Derm Venereol*. 2016;96(6):768–773.
36. Desai N, Allen J, Ali I, et al. Autoantibodies to basement membrane proteins BP180 and BP230 are commonly detected in normal subjects by immunoblotting. *Australas J Dermatol*. 2008;49(3):137–141.
37. Hachisuka H, Kurose K, Karashima T, et al. Serum from normal elderly individuals contains anti-basement membrane zone antibodies. *Arch Dermatol*. 1996;132(10):1201–1205.

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Abbreviations and Acronyms:
BM = basement membrane; **DIF** = direct immunofluorescence; **DIF+** = direct immunofluorescent positive; **DIF−** = direct immunofluorescent negative; **ELISA** = enzyme-linked immunosorbent assay; **IIF** = indirect immunofluorescence; **IgA** = immunoglobulin A; **IgG** = immunoglobulin G; **MMP** = mucous membrane pemphigoid; **SSS** = salt-split skin.

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